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(71) Applicant *(for all designated States except US)*: BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 W. 7th Street, Austin, TX 78701 (US).

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(74) Agent: WILSON, Mark, B.; Arnold, White & Durkee, P.O. Box 4433, Houston, TX 77210 (US).

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17 August 2000 (17.08.00)

(54) Title: RECOMBINANT HEPATITIS A VIRUS (HAV), HAV VARIANTS, HAV-BASED VACCINES AND METHODS OF PRODUCING THEM

(57) Abstract

The invention described herein is directed to methods and compositions involving recombinant hepatitis A virus (HAV) expressing heterologous nucleic acid sequences and a forced selection method to identify viral variants, including HAV variants, that may contain characteristics beneficial for a vaccine. Accordingly, the invention includes HAV–based vaccine virus seed and other viral vaccine seed, including methods of making them. The viruses of the present invention also generally have diagnostic uses as well as therapeutic uses for gene therapy, especially with respect to liver–specific diseases and conditions.

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INTERNATIONAL SEARCH REPORT

Inte· ıal Application No
PCT/US 99/20375

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 C12N15/36 C12N15/40 C12N15/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 478 746 A (COHEN JEFFREY I ET AL) 26 December 1995 (1995-12-26) column 6, line 46 - line 59 ---	1-3, 10-12, 14-16, 20,48-50
X	TANG S ET AL.: "Toward a poliovirus-based simian immunodeficiency virus vaccine: correlation between genetic stability and immunogenicity" JOURNAL OF VIROLOGY, vol. 71, no. 10, October 1997 (1997-10), pages 7841-7850, XP002128739 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application the whole document	48-50, 52-63
A	---	1-8, 10-16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2000	11. 05. 2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	CUPIDO, M

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INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 99/20375

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 29472 A (ALTMEYER RALF ;GIRARD MARC (FR); WERF SYLVIE V D (FR); PASTEUR INS) 22 December 1994 (1994-12-22) the whole document	48-50, 52-63
X	ZELL R ET AL: "COXSACKIEVIRUS B3 (CVB3) VARIANTS EXPRESSING CYTOKINE GENES AS A TOOL TO INFLUENCE THE LOCAL IMMUNITY IN VIVO" IMMUNOBIOLOGY, vol. 197, no. 2/04, 1997, page 336 XP002072834 the whole document	48-50, 53,62,63
A	WO 97 40166 A (US HEALTH ;RAYCHAUDHURI GOPA (US); EMERSON SUZANNE U (US); PURCELL) 30 October 1997 (1997-10-30) page 12, line 6 -page 14, line 25; figures 1,2	1-3, 10-12, 14-16, 20,40-50
A	EP 0 302 801 A (PASTEUR INSTITUT) 8 February 1989 (1989-02-08) page 2, line 4 - line 15	1-3

INTERNATIONAL SEARCH REPORT

Ir. ..tional application No.
PCT/US 99/20375

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-50, 52-63

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99/20375

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-50,52-63

Methods for producing recombinant viruses, recombinant and vaccine seed hepatitis A viruses.

2. Claims: 51, 64

Methods for producing a recombinant or vaccine seed hepatitis C virus.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

DESCRIPTION

RECOMBINANT HEPATITIS A VIRUS (HAV), HAV VARIANTS, HAV-BASED VACCINES AND METHODS OF PRODUCING THEM

Background of the Invention

This is a continuation-in-part application of United States Serial No. 60/098,945, filed on September 3, 1998, which is co-pending with the filing of this application.

I. Field of the Invention

The present invention generally relates to the fields of virology, cell biology, and molecular biology. More particularly, it is directed to methods and compositions involving recombinant hepatitis A virus (HAV) expressing heterologous nucleic acid sequences and a forced selection method to identify viral variants, including HAV variants, that may contain characteristics beneficial for a vaccine. Such viruses have diagnostic uses as well as therapeutic uses as HAV vaccine seed virus and vehicles for gene therapy, especially with respect to liver-specific diseases and conditions.

II. Description of the Related Art

The genome of wild-type Hepatitis A virus (HAV) strain HM175 has been characterized as containing 7,478 nucleotides and encoding a polypeptide of 2,227 amino acid residues. (Cohen *et al.*, 1987). A cell culture-adapted (CC) variant of strain HM175 has been cloned as a cDNA in a plasmid expression vector (Cohen *et al.*, 1989). The resultant RNA was infectious when transfected into cultured primary AGMK cells, and the virus exhibited similar growth and attenuation characteristics as the parent virus. (Cohen *et al.*, 1989, 1987).

HAV variants that are presently used for the production of inactivated HAV vaccines have been derived empirically by passage of the virus in relevant cell cultures. These viruses contain a number of mutations that enhance the growth of the virus in cell culture (compared with wild-type HAV). Some of these mutations have been studied in detail, as in the case of the HM175 strain used for production of vaccine by SmithKline Beecham. However, the repertoire of mutations which is achievable by this empiric approach appears to be limited by cytopathic effects induced by more successfully replicating HAV variants. Thus, available cell culture-adapted strains of HAV remain severely limited in their ability to replicate and produce

antigen in suitable cell lines in comparison with other viruses, such as poliovirus, a virus belonging to the same virus family as HAV (family *Picornaviridae*).

Also infectious were chimeric constructs encoding the wild-type HAV sequence with the P2/P3 sequence from the CC variant. (Cohen *et al.*, 1989). Mutations in chimeric constructs have been demonstrated to render a virus with increased efficiency of *in vitro* viral growth. (Emerson *et al.*, 1991). Accordingly, although there exist HAV genomes with favorable characteristics for production of a vaccine, methods for identifying additional variants could be used to isolate even better candidates for vaccine seed virus for commercial production of HAV vaccine.

Furthermore, a recombinant HAV containing non-HAV sequences in a polyprotein region has not been previously described. Heterologous sequences could be incorporated into the HAV genome for diagnostic and therapeutic uses, including gene therapy. Therefore, it is the object of the present invention to provide methods and compositions related to recombinant HAV and variants thereof for therapeutic and diagnostic purposes. It is another object of the present invention to provide a general selection method for the identification of viral vaccine candidates.

SUMMARY OF THE INVENTION

To overcome these and other deficiencies in the art the present invention provides a recombinant hepatitis A virus comprising a heterologous nucleic acid sequence. The invention further describes several methods for creating and screening various versions of the recombinant hepatitis A virus for use as a prophylactic and therapeutic agent against diseases.

Thus, in one embodiment, in the recombinant hepatitis A virus comprising a heterologous nucleic acid sequence, the nucleic acid sequence is comprised within a polyprotein region. In another embodiment, the recombinant hepatitis A virus comprising a heterologous nucleic acid sequence, is an attenuated virus. In one aspect of the invention, the heterologous nucleic acid sequence of the recombinant hepatitis A virus is located at the 2A/2B junction. In a related aspect, a gly-gly hinge flanks the heterologous nucleic acid sequence at the 2A/2B junction.

In a preferred embodiment of the invention, the heterologous nucleic acid sequence comprises a nucleic acid sequence encoding a selectable marker. In one embodiment, the

selectable marker is a positive selectable marker. In another embodiment, the positive selectable marker of the recombinant hepatitis A virus is a drug resistance marker. In a specific embodiment, the positive selectable marker is a phleomycin resistance gene.

In an important aspect of the invention, the heterologous nucleic acid sequence of the recombinant hepatitis virus comprises a nucleic acid sequence for treating a disease or condition. In a preferred embodiment, the disease or condition is liver-specific.

The invention also contemplates a method for producing a recombinant hepatitis A virus containing a heterologous nucleic acid sequence comprising: (a) obtaining a vector containing an infectious cDNA copy of the HAV genome; (b) cleaving the HAV genome in a polyprotein region; and (c) inserting a nucleic acid sequence comprising a heterologous nucleic acid sequence into the region. In a specific aspect, the polyprotein region comprises the 2A and 2B coding region.

In some aspects the method further comprises incubating the vector with the insertion under conditions to permit transcription. In a further aspect, the method further comprises transfecting the transcribed products into a permissive cell.

In one embodiment of the method, the heterologous nucleic acid sequence is inserted in frame. In another embodiment of the method, the heterologous nucleic acid sequence further comprises a hepatitis A virus 3C proteinase cleavage site. In yet another embodiment of the method, the heterologous nucleic acid sequence further comprises a gly-gly hinge in a flanking cleavage site. In still another embodiment of the method, the heterologous nucleic acid region comprises a selectable marker. In a further aspect of the method, the step of transfecting the transcribed product containing the heterologous nucleic acid sequence in the coding region into an HAV permissive cell is also contemplated.

The invention also describes a method for screening for a hepatitis A virus variant that comprises: (a) obtaining a recombinant hepatitis A virus comprising a selectable marker conferring resistance to a selectable agent; (b) infecting a HAV permissive cell with the recombinant HAV; and (c) exposing the infected cell to the selectable agent. In some aspects, the selectable marker is located in a polyprotein region. In another aspect, the method can further comprise the step of iterating the exposure at a higher concentration level of the selectable agent.

In one aspect, the method further comprises assaying the recombinant HAV for enhanced replication. In a related embodiment, the assay for enhanced replication comprises comparing replication foci of the cell infected with the recombinant HAV and exposed to the selectable agent with either (i) a cell infected with the recombinant HAV but not exposed to the selectable agent or (ii) a cell infected with a vector containing an infectious cDNA copy of the HAV genome and not the selectable marker but exposed to the selectable agent.

In another aspect, the method further comprises assaying the recombinant HAV for increased viral antigen production. The assay for increased viral antigen production comprises comparing viral antigen production of the cell infected with the recombinant HAV and exposed to the selectable agent with either (i) a cell infected with the recombinant HAV but not exposed to the selectable agent or (ii) a cell infected with a vector containing an infectious cDNA copy of the HAV genome and not the selectable marker but exposed to the selectable agent. In a specific embodiment of this method the viral antigen is derived from HAV. In still another aspect, the method further comprises identifying at least one mutation in a candidate variant as compared to the recombinant virus.

The invention also describes the identification of a hepatitis A virus variant by a method of screening which comprises: (a) obtaining a vector encoding a recombinant hepatitis A virus comprising a selectable marker conferring resistance to a selectable agent; (b) incubating the vector under conditions to permit transcription; (c) transfecting a HAV permissive cell with the transcribed product; and (d) exposing the infected cell to the selectable agent.

In one embodiment of the screening method, the hepatitis A virus variant is identified by a method of screening further comprising iterating the exposure at a higher concentration level of the selectable agent. In one specific aspect, the hepatitis A virus variant is identified by a method of screening further comprising assaying the recombinant HAV for enhanced replication. In another specific aspect, the variant is identified by a method of screening further comprising assaying the recombinant HAV for increased viral antigen production. In yet other specific aspects, the variant contains at least one nucleic acid sequence mutation compared to the recombinant virus. In a further aspect the nucleic acid sequence mutation is identified in the hepatitis A virus variant.

The invention also provides a method for producing a hepatitis A virus vaccine seed virus comprising: (a) obtaining a recombinant hepatitis A virus variant comprising a selectable

marker; (b) identifying at least one nucleic acid sequence mutation in the recombinant HAV variant; (c) obtaining a vector comprising an infectious cDNA copy of the virus; (d) introducing the nucleic acid sequence mutation of the recombinant HAV variant into the vector; (e) incubating the mutated vector under conditions to permit transcription; (f) infecting an HAV permissive cell with the transcribed product; and (g) incubating the infected cell under conditions to permit viral replication.

In one embodiment, the selectable marker is located in a polyprotein region. In a specific embodiment of the method, the permissive cell is an MRC-5 cell. In another specific embodiment of the method, the permissive cell is a BSC-1 cell. In yet another specific embodiment of the method, the permissive cell is a FRhK-4 cell.

The invention further describes a method where a hepatitis A virus-based vaccine seed virus is produced. The method comprises the following: (a) obtaining a recombinant hepatitis A virus variant comprising a selectable marker; (b) identifying at least one nucleic acid sequence mutation in the recombinant HAV variant; (c) obtaining a vector containing an infectious cDNA copy of the HAV genome; (d) introducing the nucleic acid sequence mutation of the recombinant HAV variant into the vector; (e) incubating the mutated vector under conditions to permit transcription; (f) infecting an HAV permissive cell with the transcribed product; and (g) incubating the infected cell under conditions to permit viral replication.

In one aspect of the method described above, the selectable marker is located in a polyprotein region. In another aspect, the hepatitis A virus variant used to produce the seed virus further comprises a heterologous nucleic acid sequence. In one aspect the hepatitis A virus variant used to produce the seed virus variant has enhanced replication. In another aspect the hepatitis A virus vaccine seed virus used to produce the seed virus variant has increased viral antigen production. In yet another aspect the heterologous nucleic acid sequence comprises a foreign antigen. In a preferred aspect of this embodiment, the foreign antigen elicits an immune response. In a specific aspect the foreign antigen is derived from the hepatitis C virus. In yet another specific aspect the foreign antigen is derived from the hepatitis B virus.

The instant invention also discloses a method for producing a recombinant virus comprising: (a) obtaining a vector comprising a viral genome, wherein said vector comprises a

heterologus nucleic acid sequence; (b) incubating the vector under conditions to permit transcription; (c) transfecting a permissive cell with the transcribed product; and (d) incubating the cell under conditions to permit viral replication. In one aspect the heterologus nucleic acid sequence is located in a polyprotein region. In another aspect, the viral genome is derived from a positive-strand RNA virus. In a specific aspect the positive strand RNA virus is a hepatitis C virus. In another aspect, the heterologous nucleic acid sequence encodes a selectable marker.

The invention also describes a method for producing a viral variant comprising: (a) obtaining a vector comprising a viral genome, wherein said vector comprises a selectable marker conferring resistance to a selectable agent; (b) incubating the vector under conditions to permit transcription; (c) transfecting a permissive cell with the transcribed product; and (d) exposing the infected cell to the selectable agent. In one aspect, the selectable marker is located in a polyprotein region. This method may further comprise iterating the exposure at a higher concentration level of the selectable agent. In one aspect this method may further comprise the step of identifying at least one mutation in a candidate variant as compared to the parent virus. In another aspect the nucleic acid sequence mutation is identified.

In some embodiments the method further comprises assaying the virus for enhanced replication. The assaying for enhanced replication can comprise comparing replication foci of the cell transfected with the vector and exposed to the selectable agent with either (i) a cell transfected with the vector but not exposed to the selectable agent or (ii) a cell transfected with a vector capable of producing a virus in a permissive cell, wherein the vector comprises nucleic acid sequences of the virus and exposed to the selectable agent.

In other embodiments, the method further comprising assaying the virus for increased viral antigen production. The assaying for increased viral antigen production comprises comparing viral antigen production of the cell transfected with the vector and exposed to the selectable agent with either (i) a cell transfected with the vector but not exposed to the selectable agent or (ii) a cell transfected with a vector capable of producing a virus in a permissive cell, wherein the vector comprises nucleic acid sequences of the virus and exposed to the selectable agent.

The invention further contemplates a method for producing a viral vaccine seed virus comprising: (a) obtaining a viral variant comprising a selectable marker; (b) identifying at

least one nucleic acid sequence mutation in the viral variant; (c) obtaining a vector comprising an infectious cDNA copy of a viral genome used to create the viral variant; (d) introducing the nucleic acid sequence mutation of the viral variant into the vector; (e) incubating the vector under conditions to permit transcription; (f) transfecting a permissive cell with the transcribed product; and (g) incubating the cell under conditions to permit viral replication. In one aspect of this method, the viral variant is derived from hepatitis C virus. In yet another aspect of this method, the viral variant is derived from hepatitis B virus.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1. The genetic organization of 18f2AZeo2B (clone #6), a recombinant HAV variant in which the *zeo* gene has been inserted in frame (with flanking 3C cleavage sites) between the 2A and 2B coding regions of the HAV genome.

FIG. 2 HAV containing the *zeo* gene at the 2A/2B junction (2AZeo2B) was used to infect BSC-1 cells cultured in increasing concentrations of Zeocin. Replication characteristics of virus harvests taken at each zeocin concentration were evaluated by radioimmunofocus assays in BSC-1 cells. As a control BSC-1 cells were infected with the parent virus HM175/18f and incubated under the same conditions. BSC-1 cells infected with HM175/18f incubated in 500μg/ml if zeocin were not viable and virus was not recovered. Larger size of replication foci in the 2azeo2 harvests taken at 500 μg/ml zeocin is indicative of enhanced replication capacity of these novel variants.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention provides compositions of recombinant HAV, recombinant variants, including HAV variants, and vaccine seed virus, including HAV-based vaccine seed virus, as well as methods of making them and using in screening assays.

Hepatitis A virus (HAV) is one of five etiologic agents that cause acute viral hepatitis in humans. It is responsible for approximately 30 percent of all reported cases of this disease in

the United States, which translates to more than 100,000 cases each year. The virus is quite contagious, being spread primarily by the fecal-oral route. Outbreaks and large-point source epidemics have been observed. While vaccines against HAV exist, the production of inactivated hepatitis A virus (HAV) vaccine is limited by the slow and inefficient growth of this virus in cultured cells.

HAV is in the Picornaviridae family, which comprises several human pathogens such as poliovirus and rhinovirus. HAV is a non-enveloped, positive-stranded RNA virus, which has at least three genotypes, but only a single serotype.

The present invention takes advantage of two discoveries. The first is the realization that HAV could accommodate a heterologous nucleic acid sequence in a polyprotein region. A heterologous nucleic acid sequence correctly positioned within the genome could behave in such a manner as to have a minimal effect on replication of the virus. Therefore, it is an object of the present invention to produce a recombinant HAV. The second discovery involved the use of a forced selection system to identify viral variants that had altered characteristics, some of which could useful for a viral vaccine. Furthermore, based on the inventors' discovery, it is a further object of the invention to provide vaccines against pathogens, such as an HAV vaccine seed virus.. Additionally, it is another object of the present invention to provide liver-specific gene therapy vectors for use in the treatment of liver diseases such as hemophilia or familiar hypercholesterolemia.

I. Recombinant Hepatitis A Virus

The introduction of non-HAV sequences into the HAV polyprotein sequence has not been previously executed. As an RNA virus, HAV is difficult to manipulate using recombinant technology. Consequently, others have cloned the HAV genome as a cDNA that is contained within an expression vector. This vector can be transcribed *in vitro* using standard techniques and the transcribed RNA products can then be transfected into permissible cells, which produce infectious virus. However, these cDNA clones do not encode HAV genomic sequences that contain a heterologous nucleic acid sequence. Before the present invention it was not known whether the HAV genome would be genetically stable with a heterologous sequence and, if it could, where the location in the genome of such a sequence could be tolerated. Recombinant HAV containing heterologous nucleic acid sequences are disclosed herein, as are methods of producing such a recombinant HAV.

A heterologous nucleic acid sequence includes any nucleic acid sequence encoding either non-HAV sequence or HAV sequence located in a position not normally found. While the recombinant HAV of the present invention can comprise any heterologous nucleic acid sequence, specific embodiments are directed at particular types of heterologous sequences.

In some embodiments of the present invention, a recombinant HAV contains a selectable marker. A selectable marker is generally a gene that encodes for a polypeptide that confers a phenotype onto a cell that expresses it. More specifically, under a particular set of conditions, a selectable gene allows for a cell expressing it to be discriminated from a cell that does not express it. The particular set of conditions frequently includes the presence of a selectable agent, which is the compound that creates the selection. For example, a drug resistance marker allows for a cell expressing it to survive under conditions in which that particular drug, the selectable agent, is present. Such a drug resistance marker operates as a positive selectable marker because cells that contain the gene are selected for, as opposed to selected against. A similar cell that does not contain the marker may not survive under those same conditions. Numerous examples of selectable markers for use in mammalian cells are known to those of skill in the art, including drug resistance markers such as the phleomycin resistance gene (*zeo*), the hygromycin resistance gene, and the *neo* gene.

In other embodiments of the present invention, a recombinant HAV or HAV variant contains a foreign antigen, which is a non-HAV encoded substance that is specifically recognized by an antibody or a T-cell receptor. An immunogen is a substance that induces an immune response, and as used herein, the terms "antigen" and "immunogen" are used interchangeably. The foreign antigen contemplated by the present invention include all or parts of viral polypeptides, other microbial polypeptides, tumor-specific antigens, or any other nucleic acid-, polypeptide-, or peptide-based epitope, against which an immune response is desired.

Also considered within the scope of the invention is a recombinant HAV or HAV variant that expresses a nucleic acid sequence encoding a sequence that would be useful in liver-specific gene therapy. Liver-specific gene therapy could be employed against a number of diseases or conditions such as liver abscess, liver cancer, cirrhosis, all forms of hepatitis, biliary atresia, cocidiodomycosis, hemochromatosis, Reye's syndrome, sclerosing cholangitis, and Wilson's disease. Examples of such sequences would include, for example, genes encoding for immunosuppressants for use in transplantations, tumor suppressor genes that

could be used for cancer therapy, vascular genes for use in cancer therapy, genes involved in therapy for hemophilia A and B, and hypercholesterolemia.

It is further contemplated that the advances made with HAV can apply to other positive strand RNA viruses, such as hepatitis C virus (HVC). Accordingly, recombinant viruses and methods of producing them are also within the scope of the invention.

II. HAV Variants

The present invention is also directed at HAV variants and methods of producing and screening for such variants. The term "variant" is used herein to refer to a virus whose genome has been altered. In some cases, a variant is identified by a phenotypic change compared to a parent virus. However, the present invention is not limited to such variants, as it also includes variants with known nucleic acid mutations that are specifically generated or randomly generated.

In some embodiments of the claimed invention, variants with enhanced replication are described, while in other embodiments, variants with increased viral antigen production are disclosed. It is contemplated that variants with other phenotypic characteristics could be created according to the claimed invention. For example, variants that have an altered host specificity or that exhibited slower growth could be produced and identified. The methods for screening for variants is not limited to variants with enhanced replication or increased viral replication. Attenuated viruses are contemplated to be within the scope of the viral variants and viral vaccines describe herein. An attenuated virus is generally one in which continuous passage of the virus in a cell, either in cell culture or in an organism, alters a property or properties of that virus such that it exhibits an altered phenotype with respect to the virus before it was continuously passaged.

As previously mentioned, the screening method employs a forced selection technique that is devised to generate variants. A selectable agent is exposed to a cell infected with a recombinant virus, such as a recombinant HAV, containing a selectable marker. Increasing concentrations of the selectable agent are then exposed to the cell as the media is iteratively changed. Such selection methods are understood by a skilled artisan. Furthermore, the techniques described above equally apply to analysis of variants. PCR may be particularly useful in characterizing variants on a genetic level. (Goswami, 1997, incorporated by reference).

The phrase "enhanced replication" is intended to be a relative term pertaining to a virus clone's ability to replicate when compared to a similar virus, such as the parent virus that is not recombinant, or the recombinant virus in the absence of selection. This comparison can be done by assaying replication foci size between a cell infected with recombinant HAV but not exposed to the selectable agent or to a cell infected with the same HAV strain that is not recombinant (parent virus) and exposed to the selectable agent. An evaluation of foci size could be undertaken by anyone of skill in the art of virology.

"Increased viral antigen production" refers to the relative amount of a viral antigen that is produced compared to a similar virus, such as the parent virus that is not recombinant, or the recombinant virus in the absence of selection. An antigen is previously described herein. A candidate antigen from HAV is VP3, as described in Pinto, 1998 and Bosch, 1998, each of which is incorporated by reference.

III. HAV Vaccine Seed Virus

The present invention also covers HAV vaccine seed virus and methods of producing such virus using recombinant HAV variants with enhanced replication. A vaccine is a pathogenic agent that can be administered prophylactically to induce immunity in an organism. A vaccine seed virus is used to produce commercially the vaccine on a large scale.

The present invention utilizes HAV variants containing a selectable marker gene that is ultimately used to create an HAV vaccine because it allows for the selection and identification of variants with altered properties, some of which could be useful in producing a vaccine. Furthermore, foreign viral antigens can placed within an HAV variant that has enhanced replication or within a recombinant HAV prior to forced selection to create a HAV-based vaccine that mostly contained HAV sequence but also included a foreign antigen. For example, insertion of hepatitis C or hepatitis B virus proteins that elicit a protective immune response, at the HAV 2A/2B junction would result in liver specific production and induction of a protective immune response. This type of vaccine would not be possible in the current setting as a killed formalin inactivated vaccine is used, however, in the advent of the development of a live attenuated this approach of a multivalent chimeric HAV vaccine is possible. Alternatively, a hepatitis viral variant could be created and used to produce a HVC live vaccine.

IV. HAV-Based Methods and Compositions

A. Polynucleotides Encoding the HAV Genome and Heterologous Sequences

Recombinant hepatitis A virus (HAV), recombinant HAV variants, and HAV vaccine seed virus of the present invention comprise an HAV virus that contains a heterologous nucleic acid sequence. Even though HAV is an RNA virus, the genome can be manipulated as a cDNA, which can then be subsequently transcribed into RNA that can be transfected into a host cell. These manipulations facilitate application of recombinant technology in creating novel HAV genomes. Furthermore the use of other viral genomes, particularly of positive strand RNA viruses , is also envisioned.

1. Nucleic acid sequences encoding the HAV genome and heterologous sequences

The present invention concerns nucleic acid sequences comprising non-HAV nucleic acid sequences or HAV sequences located at a different position than normally found that are capable of expressing a protein, polypeptide, or peptide derived from these sequences. It also involves nucleic acid sequences, both RNA and DNA, that encode parts and/or all of the HAV genome, some of which may be capable of expressing a protein, polypeptide, or peptide derived from the HAV genome. A "nucleic acid sequence" means an RNA or DNA molecule of any length. The term "polynucleotide" refers to a nucleic acid molecule, RNA or DNA, that is at least 100 nucleotides in length. "Heterologous" sequence is used to refer to a sequence not normally found in a particular context, either because it is derived from a different source, or because it is located in a different place. With HAV-based recombinant viruses, variants and vaccines, non-HAV sequences are understood to be encompassed by the term "heterologous.".

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a heterologous sequence refers to a DNA segment that contains polypeptide-coding sequences isolated away from, or purified free from, total eukaryotic, prokaryotic, and viral genomic DNA. Included within the term "DNA segment" are a polypeptide or polypeptides, DNA segments smaller than a polypeptide, and recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified heterologous gene refers to a DNA segment including non-HAV coding sequences and, in certain aspects, regulatory

sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins and mutants.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a heterologous gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

The term "cDNA" is intended to refer to DNA prepared using RNA as a template. cDNA can be prepared from either genomic DNA transcribed into mRNA, which is advantageous because the cDNA contains primarily coding sequences, or it can be prepared from genomic RNA, as is found in HAV.

As used herein in various aspects of the invention, the term "consecutive nucleic acid segment" will be understood to include a contiguous nucleic acid sequence of at least about 8, about, 9, about 10, about 11, about 12, about 13, about 14, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 260, about 270, about 280, about 290, about 300, about 310, about 320, about 330, about 340, about 350, about 360, about 370, about 380, about 390, about 400, about 410, about 420, about 430, about 440, about 450, about 460, about 470, about 480, about 490, about 500, about 600, about 700, about 800, about 900, or about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1600, about 1700, about 1800, about 1900, about 2000, about 3000, about 4000, or about 5000 nucleic acids or so.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode the HAV genome and/or a heterologous polypeptide or peptide that includes within its amino acid sequence a contiguous

amino acid sequence in accordance with, or essentially corresponding to the named polypeptides

The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids. For optimization of expression of HAV polypeptides or heterologous polypeptides in human cells, the codons are shown in preference of use from left to right, in Table 1. The most preferred codon for alanine is thus "GCC," and the least is "GCG" (see Table 1, below).

TABLE 1

Preferred Human DNA Codons

Amino Acids				**Codons**					
Alanine	Ala	A		GCC	GCT	GCA	GCG		
Cysteine	Cys	C		TGC	TGT				
Aspartic acid	Asp	D		GAC	GAT				
Glutamic acid	Glu	E		GAG	GAA				
Phenylalanine	Phe	F		TTC	TTT				
Glycine	Gly	G		GGC	GGG	GGA	GGT		
Histidine	His	H		CAC	CAT				
Isoleucine	Ile	I		ATC	ATT	ATA			
Lysine	Lys	K		AAG	AAA				
Leucine	Leu	L		CTG	CTC	TTG	CTT	CTA	TTA
Methionine	Met	M		ATG					
Asparagine	Asn	N		AAC	AAT				
Proline	Pro	P		CCC	CCT	CCA	CCG		
Glutamine	Gln	Q		CAG	CAA				
Arginine	Arg	R		CGC	AGG	CGG	AGA	CGA	CGT
Serine	Ser	S		AGC	TCC	TCT	AGT	TCA	TCG
Threonine	Thr	T		ACC	ACA	ACT	ACG		
Valine	Val	V		GTG	GTC	GTT	GTA		
Tryptophan	Trp	W		TGG					
Tyrosine	Tyr	Y		TAC	TAT				

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5′ or 3′ sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences

flanking either of the 5′ or 3′ portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, enhancers, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

For example, nucleic acid fragments may be prepared that include a contiguous stretch of nucleotides identical to or complementary to the known sequence for the HAV genome or a heterologous nucleic acid, such as about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, about 100, about 125, about 150, about 250, about 300, about 400, about 600, about 750, about 800 or about 900 nucleotides, and that are up to about 1,000,000, about 750,000, about 500,000, about 250,000, about 100,000, about 50,000, about 20,000, or about 10,000, or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases. In certain cases, nucleotide segments of a million bases or more, including chromosome sized pieces of DNA, are contemplated as being useful. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*;

including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002, 15,000, 20,000 and the like.

The nucleic acid segments used in the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

In addition to the "standard" DNA and RNA nucleotide bases, modified bases are also contemplated for use in particular applications of the present invention. A table of exemplary, but not limiting, modified bases is provided herein below.

Table 2 - Modified Bases

Abbr.	Modified base description	Abbr.	Modified base description
ac4c	4-acetylcytidine	mam5s2 u	5-methoxyaminomethyl-2-thiouridine
chm5u	5-(carboxyhydroxylmethyl)uridine	man q	beta,D-mannosylqueosine
cm	2'-O-methylcytidine	mcm5s2 u	5-methoxycarbonylmethyl-2-thiouridine
cmnm5s2 u	5-carboxymethylaminomethyl-2-thioridine	mcm5u	5-methoxycarbonylmethyluridine
cmnm5u	5-carboxymethylaminomethyluridine	mo5u	5-methoxyuridine
d	dihydrouridine	ms2i6a	2-methylthio-N6-isopentenyladenosine
fm	2'-O-methylpseudouridine	ms2t6a	N-((9-beta-D-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine
gal q	beta,D-galactosylqueosine	mt6a	N-((9-beta-D-ribofuranosylpurine-6-yl)N-methyl-carbamoyl)threonine

Table 2 – Continued

gm	2'-O-methylguanosine	mv	uridine-5-oxyacetic acid methylester
i	inosine	o5u	uridine-5-oxyacetic acid (v)
i6a	N6-isopentenyladenosine	osyw	wybutoxosine
m1a	1-methyladenosine	p	pseudouridine
m1f	1-methylpseudouridine	q	queosine
m1g	1-methylguanosine	s2c	2-thiocytidine
m1i	1-methylinosine	s2t	5-methyl-2-thiouridine
m22g	2,2-dimethylguanosine	s2u	2-thiouridine
m2a	2-methyladenosine	s4u	4-thiouridine
m2g	2-methylguanosine	t	5-methyluridine
m3c	3-methylcytidine	t6a	N-((9-beta-D-ribofuranosylpurine-6-yl)carbamoyl)threonine
m5c	5-methylcytidine	tm	2'-O-methyl-5-methyluridine
m6a	N6-methyladenosine	um	2'-O-methyluridine
m7g	7-methylguanosine	yw	wybutosine
mam5u	5-methylaminomethyluridine		3-(3-amino-3-carboxypropyl)uridine
	5-fluorouracil		hypoxanthine
	5-bromouracil		xanthine
	5-chlorouracil		(acp3)w
	5-iodouracil		2,6-diaminopurine
	N6-adenine		

2. Mutagenesis

Although some of the embodiments of the present invention rely on random mutagenesis of the HAV genome, site-specific mutagenesis also has use in the present invention. For example, some mutations are known to confer desirable characteristics, for example, high titer growth in cell culture, onto the wild-type HAV genome. *See* U.S. Patent

No. 5,478,746. The wild-type genome could be specifically mutagenized at particular places in the HAV genome, and then the mutagenized genome could be used in the claimed methods of the present invention to identify recombinant HAV and HAV variants with even more enhanced characteristics. Site-specific mutagenesis is a technique useful in the preparation of proteins or peptides with essentially the same function as the non-mutant nucleic acid sequence, but with alterations in the activity of the protein or peptide, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

The technique of site-specific mutagenesis is generally well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a polynucleotide sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

3. Vectors

The present invention uses vectors comprising DNA sequences to produce recombinant HAV, recombinant HAV variants, and HAV vaccine seed virus. cDNA from the HAV RNA genome has been made and placed into vectors that can then be manipulated by standard recombinant methodologies (U.S. Patent No. 5,837,260 and 5,478,746, herein incorporated by reference). The term "vector" is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.,* YACs). The term "expression vector" refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. Expression vectors can contain a variety of "control sequences," which refers to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism.

These control sequences may be obtained from any prokaryote, eukaryote, or virus, and they may be joined to other sequences using standard molecular biology techniques. For example, restriction enzymes may be employed to digest or cleave a nucleic acid sequence at a particular location. In one embodiment of the claimed invention, for example, the HAV genome is cleaved between the 2A and 2B coding regions. Once cleaved, exogenous or heterologous nucleic acid sequences can be ligated to or joined at the cleavage sites.

a. Promoters and Enhancers

A "promoter" is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases "operatively positioned," "operatively linked," "under control," and "under transcriptional control" mean that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or

expression of that sequence. A promoter may or may not be used in conjunction with an "enhancer," which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant and/or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant and/or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and/or promoters or enhancers isolated from any other prokaryotic, viral, and/or eukaryotic cell, and/or promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Patent 4,683,202, U.S. Patent 5,928,906, each incorporated herein by reference).

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the DNA segment in the cell type and organism chosen for expression. In the present invention, HAV promoters and/or enhancers can be used to direct proper expression of the HAV gene products; alternatively, liver-specific promoters or enhancer may be employed to direct the expression of a heterologous nucleic acid sequence for use in liver-specific gene therapy. The use of promoters, enhancers, and/or cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. Furthermore, promoters may be employed that are compatible with *in vitro* transcription

techniques, such as the T3, T7, and SP6 promoters. RNA transcription kits that can be used to transcribe an expression construct containing at least one of these promoters are available commercially and are well known to those of skill in the art. In some embodiments of the present invention expression constructs containing a T3, T7, or SP6 promoter are use to transcribe HAV cDNA sequences into RNA that can be transfected into a host cell. The term "transcribed product" refers to an RNA molecule that is the production of a transcription reaction, which includes *in vitro* reactions.

Tables 2 lists several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 3 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

TABLE 3

Promoter and/or Enhancer

Promoter/Enhancer	References
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al*, 1984; Kiledjian *et al*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and/or Baltimore, 1983; Picard and/or Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and/or DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase (MCK)	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988

TABLE 3

Promoter and/or Enhancer

Promoter/Enhancer	References
Elastase I	Omitz *et al.*, 1987
Metallothionein (MTII)	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989

Table 3 – Continued

t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
c-fos	Cohen *et al.*, 1987
c-HA-*ras*	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse and/or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor (PDGF)	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh *et al.*, 1985; Firak *et al.*, 1986; Herr *et al.*, 1986; Imbra *et al.*, 1986; Kadesch *et al.*, 1986; Wang *et al.*, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and/or Villarreal, 1988
Retroviruses	Kriegler *et al.*, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander *et al.*, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman *et al.*, 1989

Table 3 – Continued

Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and/or Wilkie, 1983; Spalholz *et al.*, 1985; Lusky *et al.*, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens *et al.*, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and/or Siddiqui, 1986; Jameel and/or Siddiqui, 1986; Shaul *et al.*, 1987; Spandau *et al.*, 1988; Vannice *et al.*, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber *et al.*, 1988; Jakobovits *et al.*, 1988; Feng *et al.*, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp *et al.*, 1989; Braddock *et al.*, 1989
Cytomegalovirus (CMV)	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking *et al.*, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 4

Inducible Elements

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and/or Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors *et al.*, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	ElA	Imperiale *et al.*, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b

Table 4 – Continued

Murine MX Gene	Interferon, Newcastle Disease Virus	Hug *et al.*, 1988
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	EIA, SV40 Large T Antigen	Taylor *et al.*, 1989, 1990a, 1990b
Proliferin	Phorbol Ester-TPA	Mordacq *et al.*, 1989
Tumor Necrosis Factor	PMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human LIMK2 gene (Nomoto *et al.* 1999), the somatostatin receptor 2 gene (Kraus *et al.*, 1998), murine epididymal retinoic acid-binding gene (Lareyre *et al.*, 1999), human CD4 (Zhao-Emonet *et al.*, 1998), mouse alpha2 (XI) collagen (Tsumaki, *et al*, 1998), D1A dopamine receptor gene (Lee, *et al.*, 1997), insulin-like growth factor II (Wu *et al*, 1997), human platelet endothelial cell adhesion molecule-1 (Almendro *et al.*, 1996). A liver-specific promoter could be particularly useful in the context of the present invention to drive the expression of the HAV genome and/or a heterologous nucleic acid sequence.

b. Polyadenylation signals

Where a cDNA insert is employed in the present invention to encode a heterologous nucleic acid sequence, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

c. Selectable markers in the vector backbone

While some aspects of the claimed invention such as a recombinant HAV comprise a selectable marker, selectable markers are also useful to identify vector clones. For example, a vector could encode the amp^r gene, which confers resistance to the antibiotic ampicillin. Bacteria can be transformed with this vector, and positive transformants can be selected on ampicillin-containing media. In certain embodiments of the invention, the cells contain nucleic acid construct of the present invention, a cell may be identified *in vitro* or *in vivo* by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Usually the inclusion of a drug selection marker aids in cloning and in the selection of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) or chloramphenicol acetyltransferase (CAT) may be employed. Immunologic markers also can be employed. The selectable marker employed in the vector backbone is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable markers are well known to one of skill in the art.

d. Multigene constructs and IRES

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several

proteins can be simultaneously engineered into a cell with a single vector and a single selectable marker.

e. Other Sequences

In addition, non-regulatory sequences may also be added to a vector to alow the resulting vector to be manipulated. For example in one embodiment of the claimed invention a proteinase cleavage site, such as the HAV 3C proteinase cleavage site located in a position not normally found with respect to the HAV genome, is ligated into the genome. Other proteinase cleavage sites could be utilized to allow polypeptides to be easily cleaved, as is sometimes the case with fusion proteins. Alternatively, linkers could be inserted into a nucleic acid sequence. Linkers include restriction enzyme linkers as well as linkers such as a gly-gly hinge that confers flexibility in any polypeptide containing it.

f. Viral vectors

The present invention employs expression constructs that encode the HAV genome. The constructs are introduced into permissive cells to enable the production of a functional Hepatitis A Virus, which is infectious. Alternatively, the description of viral vectors is provided to disclose ways of constructing viral vectors such as an HAV viral vector for use in gene therapy (see also U.S. Patent Nos. 5,670,488 and 5,856,152, incorporated herein by reference).

There are a number of ways in which expression vectors may be introduced into cells. In certain embodiments of the invention, the expression vector comprises a virus or engineered vector derived from a viral genome. The development of viral vectors is an improvement to the field of gene transfer, as demonstrated by U.S. Patent Application No. 60/078205, hereby incorporated by reference. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986).

In addition to the use of herpes simplex virus, another method for delivery involves the use of an adenovirus expression vector. "Adenovirus expression vector" is meant to include those vectors containing adenovirus sequences sufficient to (a) support packaging of the vector and (b) to express a polynucleotide that has been cloned therein. In this context, expression may require that the gene product be synthesized.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are *cis* elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.,* 1991; Gomez-Foix *et al.,* 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet *et al.,* 1990; Rich *et al.,* 1993). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.,* 1991; Rosenfeld *et al.,* 1992), muscle injection (Ragot *et al.,* 1993), peripheral intravenous injections (Herz and Gerard, 1993) and stereotactic inoculation into the brain (Le Gal La Salle *et al.,* 1993).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene contains a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5′ and 3′ ends of the viral genome. These contain strong promoter and enhancer sequences and also are required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann *et al.,* 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for

example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. These different viral vectors offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al*, 1988; Horwich *et al.*, 1990).

g. Non-viral methods of gene transfer

Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression vector has been delivered into the cell the nucleic acid encoding the gene of interest may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression vector is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression vector employed.

In yet another embodiment of the invention, the expression vector may simply consist of naked recombinant DNA or plasmids. Transfer of the vector may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of calcium phosphate precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of calcium phosphate-precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a gene of interest also may be transferred in a similar manner *in vivo* and express the gene product.

In still another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, *i.e.*, *ex vivo* treatment. Again, DNA encoding a particular heterologous gene, or fragments thereof, may be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression vector may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.*, (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau *et al.*, (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA vector, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Other expression vectors that can be employed to deliver a nucleic acid encoding a particular gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). A synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) also has been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.*, (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the

insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a particular gene also may be specifically delivered into a cell type such as lung, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, epidermal growth factor (EGF) may be used as the receptor for mediated delivery of a nucleic acid encoding a gene in many tumor cells that exhibit upregulation of EGF receptor. Mannose can be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as targeting moieties.

In certain embodiments, gene transfer may more easily be performed under *ex vivo* conditions. *Ex vivo* gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells *in vitro*, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues.

Continuous perfusion of an expression vector or a viral vector also is contemplated. The amount of vector or peptide delivered in continuous perfusion can be determined by the amount of uptake that is desirable.

h. Transfected cell cultures and cell lines

In some embodiments the present invention utilizes expression constructs encoding the HAV genome to produce an infectious recombinant HAV. The constructs are introduced into cells, cell cultures, or cell lines. While various mammalian host cell lines such as HeLa, HepG2, 3T3, MDCK can be used, cells most frequently used with the current invention are cells permissive for HAV. A permissive cell is one that supports the replication of HAV and that frequently, as a consequence, undergoes lysis. These lines include MRC-5, a diploid human line suitable for production of human vaccines, Vero cells, BSC-1, and FRhK-4. Furthermore, the present invention contemplates that these permissive cells could be used for infection by recombinant HAV virus for a number of purposes, including the use of these cells to assay HAV activity.

Primary mammalian cell cultures may be prepared in various ways. In order for the cells to be kept viable while *in vitro* and in contact with the expression vector, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshney, 1992). Furthermore, the present

invention sometimes requires that the cells be cultured under conditions that permit HAV replication. Such conditions are well known to those of skill in the art.

Also well known to those of skill in the art is the preparation of conditions that allow for selection of a cell such as resistance to a drug. In some embodiments of the present invention, increasing concentration levels of a drug in media are exposed to cells, some of which may contain a drug resistance marker. Cells expressing that marker can survive certain concentration levels, but there may become a point at which the concentration level increases so much that only cells that mutate to accommodate the higher levels are viable. A skilled artisan would be able to increase the concentration in increments that permits such mutations to occur.

4. Cloning heterologous nucleic acid sequences

The present invention contemplates cloning heterologous nucleic acid sequences including genes or cDNAs from mammalian tissue or cells for use in recombinant HAV, HAV variants, and HAV vaccine seed virus. A technique often employed by those skilled in the art of protein production today is to obtain a so-called "recombinant" version of the protein, to express it in a recombinant cell and to obtain the protein from such cells. These techniques are based upon the cloning of a DNA molecule encoding the protein from a DNA library, *i.e.*, on obtaining a specific DNA molecule distinct from other portions of DNA. This can be achieved by, for example, cloning a cDNA molecule, or cloning a genomic-like DNA molecule.

The first step in such cloning procedures is the screening of an appropriate DNA library, for example, human liver cells. The screening protocol may utilize nucleotide segments or probes that are designed to hybridize to cDNA or genomic sequences of a particular heterologous nucleic acid of interest. Additionally, antibodies designed to bind to the expressed heterologous proteins, polypeptides, or peptides may be used as probes to screen an appropriate DNA expression library. Alternatively, activity assays may be employed. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, in Sambrook *et al.* (1989), incorporated herein by reference. Moreover, as the present invention encompasses the cloning of genomic segments as well as cDNA molecules, it is contemplated that suitable genomic cloning methods, as known to those in the art, may also be used.

As used herein "designed to hybridize" means a sequence selected for its likely ability to hybridize to the heterologous nucleic acid sequence of interest, for example due to a expected high degree of homology between another organism and the desired human genes. Also included are segments or probes altered to enhance their ability to hybridize to or bind to a heterologous sequence of choice. Additionally, these regions of homology also include amino acid sequences of 4 or more consecutive amino acids selected and/or altered to increase conservation of the amino acid sequences in comparison to the same or similar region of residues in the same or related genes in one or more species. The term "designed to bind" in refers to an antibody that is selected for its likely ability to bind the desired heterologous protein, polypeptides, peptides, or antigenic fragments thereof, particularly during a screening assay for the target gene, including amino acids altered to increase antigenicity.

5. Nucleic acid detection methods

In addition to the recombinant techniques previously described, the present invention may also involve the use of various nucleic acid detection methods. Such methods could be used, for example, to identify whether a heterologous nucleic acid sequence is contained within a particular recombinant HAV or to characterize an HAV variant that has enhanced growth properties by identifying any mutations in the HAV genome. Nucleic acid detection methods are well known to those of skill in the art. Some of these methods are presented below.

a. Hybridization

The use of a hybridization probe of between 17 and 100 nucleotides in length, or in some aspect of the invention even up to 1-2 Kb or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over stretches greater than 20 bases in length are generally preferred, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of particular hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having stretches of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production. The use if hybridization can be used to confirm the presence of a particular sequence. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence.

For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating specific genes or detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications, for example, substitution of nucleotides by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, at temperatures ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe molecules, hybridization is detected, or even quantified, by means of the label.

b. Amplification and PCR™

Nucleic acid used as a template for amplification is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR™) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, each incorporated herein by reference in entirety. The use of other nucleic acid amplification reaction are contemplated, including ligase chain reaction (LCR), disclosed in EPA No. 320 308, incorporated herein by reference in its entirety, Qbeta Replicase, described in PCT Application No. PCT/US87/00880, incorporated herein by reference, Strand Displacement Amplification (SDA), Repair Chain Reaction (RCR), and other amplification methods described in GB Application No. 2 202 328 and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety. Additional nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference). Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a different nucleic acid amplification process, which may be used in accordance with the present invention.

c. Detection of PCR products and nucleic acids generally

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, for example, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989.

One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

Other methods for genetic screening to accurately detect mutations in genomic DNA, cDNA or RNA samples may be employed, depending on the specific situation. Historically, a number of different methods have been used to detect point mutations, including denaturing gradient gel electrophoresis (DGGE), restriction enzyme polymorphism analysis, chemical and enzymatic cleavage methods, and others. The more common procedures currently in use include direct sequencing of target regions amplified by PCR™ (see above) and single-strand conformation polymorphism analysis (SSCP).

Another method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA and RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single and multiple base point mutations.

U.S. Patent No. 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. After the RNase cleavage reaction, the

RNase is inactivated by proteolytic digestion and organic extraction, and the cleavage products are denatured by heating and analyzed by electrophoresis on denaturing polyacrylamide gels. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

Currently available RNase mismatch cleavage assays, including those performed according to U.S. Patent No. 4,946,773, require the use of radiolabeled RNA probes. Myers and Maniatis in U.S. Patent No. 4,946,773 describe the detection of base pair mismatches using RNase A. Other investigators have described the use of an *E. coli* enzyme, RNase I, in mismatch assays. Because it has broader cleavage specificity than RNase A, RNase I would be a desirable enzyme to employ in the detection of base pair mismatches if components can be found to decrease the extent of non-specific cleavage and increase the frequency of cleavage of mismatches. The use of RNase I for mismatch detection is described in literature from PROMEGA® Biotech. PROMEGA®markets a kit containing RNase I that is shown in their literature to cleave three out of four known mismatches, provided the enzyme level is sufficiently high.

The RNase protection assay was first used to detect and map the ends of specific mRNA targets in solution. The assay relies on being able to easily generate high specific activity radiolabeled RNA probes complementary to the mRNA of interest by *in vitro* transcription. Originally, the templates for *in vitro* transcription were recombinant plasmids containing bacteriophage promoters. The probes are mixed with total cellular RNA samples to permit hybridization to their complementary targets, then the mixture is treated with RNase to degrade excess unhybridized probe. Also, as originally intended, the RNase used is specific for single-stranded RNA, so that hybridized double-stranded probe is protected from degradation. After inactivation and removal of the RNase, the protected probe (which is proportional in amount to the amount of target mRNA that was present) is recovered and analyzed on a polyacrylamide gel.

The RNase protection assay was adapted for detection of single base mutations. In this type of RNase A mismatch cleavage assay, radiolabeled RNA probes transcribed *in vitro* from wild-type sequences, are hybridized to complementary target regions derived from test samples. The test target generally comprises DNA (either genomic DNA or DNA amplified by cloning

in plasmids or by PCR™), although RNA targets have occasionally been used. If single nucleotide (or greater) sequence differences occur between the hybridized probe and target, the resulting disruption in Watson-Crick hydrogen bonding at that position ("mismatch") can be recognized and cleaved in some cases by single-strand specific ribonuclease. To date, RNase A has been used almost exclusively for cleavage of single-base mismatches, although RNase I has recently been shown as useful also for mismatch cleavage. There are recent descriptions of using the MutS protein and other DNA-repair enzymes for detection of single-base mismatches.

Detection methods for nucleic acids can be used to assay various cellular and molecular properties of a cell or a virus. One of skill in the art would know how to use the methods described herein to detect enhanced growth. The amount of nucleic acid molecules during a particularly time frame could be easily determined using nucleic acid amplification techniques.

B. Proteins, Polypeptides, and Peptides

Through the use of recombinant HAV, the present invention covers the expression of proteins, polypeptides, or peptides encoded by the HAV genome and heterologous nucleic acid sequences. The term "purified" proteins, polypeptides, or peptides as used herein, is intended to refer to a proteinaceous composition, isolatable from mammalian cells or recombinant host cells, wherein the protein, polypeptide, or peptide is purified to any degree relative to its naturally-obtainable state, *i.e.*, relative to its purity within a cellular extract. A purified protein, polypeptide, or peptide therefore also refers to a wild-type or mutant protein, polypeptide, or peptide free from the environment in which it naturally occurs.

The term "polyprotein region" is used to refer to a region that encodes at least one gene product, as opposed to a noncoding region. The junction between 2A and 2B in teh HAV genome is an example of a polyprotein region.

Encompassed by the invention are proteinaceous segments of relatively small peptides, such as, for example, peptides of from about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 35, about 40, about 45, to about 50 amino acids in length, and more preferably, of from about 15 to about 30 amino acids in length, and also larger polypeptides of from about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 65, about 70, about 75, about 80, about 85, about 90, about

95, about 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 750, about 800, about 850, about 900, about 950, about 1000, and about 1100, up to and including proteins corresponding to the full-length sequences.

Various methods for quantifying the degree of purification of proteins, polypeptides, or peptides will be known to those of skill in the art (see generally, Ausubel, 1994, incorporated by reference). These include, for example, determining the specific protein activity of a fraction, or assessing the number of polypeptides within a fraction by gel electrophoresis. Assessing the number of polypeptides within a fraction by SDS/PAGE analysis will often be preferred in the context of the present invention as this is straightforward.

To purify a protein, polypeptide, or peptide a natural or recombinant composition, proteins, polypeptides, or peptides will be subjected to fractionation to remove various components not of interest from the composition. In addition to those techniques described in detail herein below, various other techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite, lectin affinity and other affinity chromatography steps; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques.

Another example is the purification of particular fusion protein using a specific binding partner. Such purification methods are routine in the art. This is exemplified by the generation glutathione S-transferase fusion protein, expression in *E. coli*, and isolation to homogeneity using affinity chromatography on glutathione-agarose or the generation of a polyhistidine tag on the N- or C-terminus of the protein, and subsequent purification using Ni-affinity chromatography.

In some embodiments of the claimed invention, a foreign antigen is encoded by a recombinant HAV. Alternatively, the recombinant HAV encodes at least one epitope of an antigen. U.S. Patent 4,554,101, (Hopp) incorporated herein by reference, teaches the identification and preparation of epitopes from primary amino acid sequences on the basis of hydrophilicity. Through the methods disclosed in Hopp, one of skill in the art would be able to

identify epitopes from within an amino acid sequence such as Us1.5 sequences disclosed herein in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

Numerous scientific publications have also been devoted to the prediction of secondary structure, and to the identification of epitopes, from analyses of amino acid sequences (Chou and Fasman, 1974a,b; 1978a,b, 1979). Any of these may be used, if desired, to supplement the teachings of Hopp in U.S. Patent 4,554,101.

Moreover, computer programs are currently available to assist with predicting antigenic portions and epitopic core regions of proteins. Examples include those programs based upon the Jameson-Wolf analysis (Jameson and Wolf, 1988; Wolf *et al.*, 1988), the program PepPlot® (Brutlag *et al.*, 1990; Weinberger *et al.*, 1985), and other new programs for protein tertiary structure prediction (Fetrow and Bryant, 1993). Another commercially available software program capable of carrying out such analyses is MacVector (IBI, New Haven, CT).

In further embodiments, major antigenic determinants of a polypeptide may be identified by an empirical approach in which portions of the gene encoding the polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, PCR™ can be used to prepare a range of peptides lacking successively longer fragments of the C-terminus of the protein. The immunoactivity of each of these peptides is determined to identify those fragments or domains of the polypeptide that are immunodominant. Further studies in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide to be more precisely determined.

Another method for determining the major antigenic determinants of a polypeptide is the SPOTs™ system (Genosys Biotechnologies, Inc., The Woodlands, TX). In this method, overlapping peptides are synthesized on a cellulose membrane, which following synthesis and deprotection, is screened using a polyclonal or monoclonal antibody. The antigenic determinants of the peptides which are initially identified can be further localized by performing subsequent syntheses of smaller peptides with larger overlaps, and by eventually replacing individual amino acids at each position along the immunoreactive peptide.

Once one or more such analyses are completed, polypeptides are prepared that contain at least the essential features of one or more antigenic determinants. The peptides are then employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants can also be constructed and inserted into expression vectors by standard methods, for example, using PCR™ cloning methodology.

The use of such small peptides for antibody generation or vaccination typically requires conjugation of the peptide to an immunogenic carrier protein, such as hepatitis B surface antigen, keyhole limpet hemocyanin or bovine serum albumin. Methods for performing this conjugation are well known in the art.

C. Immunodetection Methods

The present invention utilizes different recombinant HAV constructs, variants and vaccine seed virus. In some embodiments of the present invention, assays to identify or characterize the presence of a particular compound are desirable, for example, to determine whether a construct is properly expressing a heterologous gene. Accordingly, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biological components such as HAV or heterologous polypeptides, peptides, or proteins. Antibodies may be employed to detect HAV, or alternatively, antibodies could be used to identify and quantify the level of antigen that a viral variant or vaccine seed virus could produce. Furthermore, in some of the embodiments of the present invention immunoassays are employed in combination with an HAV variant that has increased viral antigen production for diagnostic assays. The specific use of diagnostic assays involving HAV antigens is described in LaBrecque, 1998, which creates a recombinant HAV antigen. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Nakamura *et al.* (1987), incorporated herein by reference.

In general, the immunobinding methods include obtaining a sample suspected of containing the desired protein, polypeptide or peptide, and contacting the sample with a first antibody in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

The immunobinding methods also include methods for detecting or quantifying the amount of a protein reactive component in a sample, which methods require the detection or quantification of any immune complexes formed during the binding process. Here, one would

obtain a sample suspected of containing the protein or peptide of interest, and contact the sample with an antibody against it, and then detect or quantify the amount of immune complexes formed under the specific conditions.

Contacting the chosen biological sample with the antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time lone enough for the antibodies to form immune complexes with, *i.e.*, to bind to any protein antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological or enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

The antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

1. ELISAs

As detailed above, immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, the appropriate antibodies are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the antigen of interest is added to the wells. After binding and washing to remove non-specifically bound immune complexes, the bound protein antigen may be detected. Detection is generally achieved by the addition of a secondary antibody recognizing the first antibody and that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA." Detection may also be achieved by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

Other types of ELISA experiments are known to those of skill in the art. Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described in more detail below.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period

of h. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 h or so, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase,

alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 h at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS) and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, *e.g.*, using a visible spectra spectrophotometer.

The immunoassays described herein could be implemented to detect and quantify viral antigen production. Similarly, these methods could be used to detect whether viral replication occurs more quickly by targetting a number of marker proteins that are affected by viral replication.

2. Immunohistochemistry

To assay proteins, polypeptides, and peptides utilized in the claimed invention, ntibodies may also be used in conjunction with both fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

D. Biological Functional Equivalents

As modifications and changes may be made in the structure of HAV genes and proteins, as well as heterologous nucleic acid sequences and proteins, of the present invention, and still allow molecules having like or otherwise desirable characteristics to be obtained, such biologically functional equivalents are also encompassed within the present invention.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies, binding sites on substrate molecules or receptors, or such like. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be

made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. It is thus contemplated that various changes may be made in the sequence HAV proteins, polypeptides or peptides, or the underlying nucleic acids, without appreciable loss of their biological utility or activity.

In terms of functional equivalents, it is well understood by the skilled artisan that, inherent in the definition of a "biologically functional equivalent" protein or peptide or gene, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted.

In particular, where shorter length peptides are concerned, it is contemplated that fewer amino acids changes should be made within the given peptide. Longer domains may have an intermediate number of changes. The full length protein will have the most tolerance for a larger number of changes. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention. It is also well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, *e.g.*, residues in binding regions or active sites, such residues may not generally be exchanged. In this manner, functional equivalents are defined herein as those peptides which maintain a substantial amount of their native biological activity.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

To effect more quantitative changes, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine

(+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as in certain embodiments of the present invention. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA; taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid. A table of amino acids and their

codons is presented herein above for use in such embodiments, as well as for other uses, such as in the design of probes and primers and the like.

In addition to the HAV and heterologous compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. Certain mimetics that mimic elements of protein secondary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orientate amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

Some successful applications of the peptide mimetic concept have focused on mimetics of β-turns within proteins, which are known to be highly antigenic. Likely β-turn structure within a polypeptide can be predicted by computer-based algorithms, as discussed herein. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

The generation of further structural equivalents or mimetics may be achieved by the techniques of modeling and chemical design known to those of skill in the art. The art of receptor modeling is now well known, and by such methods a chemical that binds to enamel matrix serine proteinase, enamelysin or enamelin can be designed and then synthesized. It will be understood that all such sterically designed constructs fall within the scope of the present invention.

In addition to the 20 "standard" amino acids provided through the genetic code, modified or unusual amino acids are also contemplated for use in the present invention. A table of exemplary, but not limiting, modified or unusual amino acids is provided herein below.

TABLE 5

Modified and Unusual Amino Acids

Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine

TABLE 5

Modified and Unusual Amino Acids

Abbr.	Amino Acid	Abbr.	Amino Acid
bAad	3- Aminoadipic acid	Hyl	Hydroxylysine
bAla	β-alanine, β-Amino-propionic acid	aHyl	allo-Hydroxylysine
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	aIle	allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
bAib	3-Aminoisobutyric acid	MeIle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

E. Virus Growth and Maintenance

Some of the embodiments of the present invention involve a recombinant HAV that contains a heterologous sequence and methods of making the recombinant HAV. While recombinant technology can be utilized to create a vector that itself is used to produce HAV, common virology techniques and methods may also be required to practice the claimed invention. For example, methods of creating an attenuated, cell culture-adapted version of HAV can be created using a vector encoding the HAV genome according to methods known to those of skill in the art, according to U.S. Patent No. 5,837,260 and 5,478,746, each incorporated by reference. While propagation of a recombinant HAV may require that the vector be introduced or transfected into a cell, particularly a permissive one, conditions that effect propagation and/or replication of the virus may be employed. Such conditions are well known to those of skill in the art.

Once a recombinant virus is obtained, the virus can be propagated by directly infecting a permissive cell under conditions to effect replication. A skilled virologist would be familiar with techniques that can be used to determine factors influencing infectivity such as multiplicity of infection (MOI), as well as those that can be used to evaluate efficacy of infection, such as the number of particle forming units (pfu).

Where the term "substantially purified" is used with respect to a recombinant HAV, this will refer to a composition in which recombinant HAV forms the major component of the composition, such as constituting about 50% of the proteins in the composition or more. In preferred embodiments, a substantially purified virus will constitute more than 60%, 70%, 80%, 90%, 95%, 99% or even more of the proteins in the composition.

A virus that is "purified to homogeneity," as applied to the present invention, means that the peptide, polypeptide or protein has a level of purity where the peptide, polypeptide or protein is substantially free from other proteins and biological components. For example, a purified peptide, polypeptide or protein will often be sufficiently free of other protein components so that degradative sequencing may be performed successfully.

F. Formulations and Routes for Administration to Patients of HAV-Based Compositions

Where clinical applications are contemplated as with the vaccine preparations and viral gene therapy preparations, it will be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks, polypeptides, peptides, antibodies, and drugs--in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render delivery vectors stable and allow for uptake by target cells. Buffers also will be employed when recombinant cells are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the vector to cells, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrase "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, "pharmaceutically

acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well know in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The active compositions of the present invention may include classic pharmaceutical preparations. Administration of these compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration may be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions, described *supra*.

The expression vectors and delivery vehicles also may be administered parenterally or intraperitoneally. Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert

gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) immunity to HAV or (ii) treatment of a liver-specific condition or disease. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

The recombinant hepatitis A viruses of the present invention may be administered directly into animals and humans, or alternatively, administered to cells that are subsequently administered to these organisms. The viruses can be combined with the various β-interferon inhibiting formulations to produce transducing formulations with greater transduction efficiencies.

1. Liposomes and nanocapsules

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of recombinant HAV, protein, polypeptides, peptides or agents, or gene therapy vectors into host cells. The formation and use of liposomes is generally known to those of skill in the art, and is also described below.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable

polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

The following information may also be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one may operate at the same time.

2. *in vitro, ex vivo, in vivo* administration

As used herein, the term *in vitro* administration refers to manipulations performed on cells removed from an animal, including, but not limited to, cells in culture. The term *ex vivo* administration refers to cells which have been manipulated *in vitro*, and are subsequently

administered to a living animal. The term in vivo administration includes all manipulations performed on cells within an animal.

In certain aspects of the present invention, the compositions may be administered either *in vitro*, *ex vivo*, or *in vivo*. In certain *in vitro* embodiments, hepatocytes are incubated with a recombinant HAV of the instant invention for 24 to 48 hours. The transduced cells can then be used for *in vitro* analysis, or alternatively for *in vivo* administration.

In vivo administration of the compositions of the present invention also are contemplated. Examples include, but are not limited to, transduction of liver cells by infusion of appropriate transducing compositions through the portal vein *via* a catheter (Bao *et al.*, 1996). Additional examples include direct injection of tumors with the instant transducing compositions, and either intranasal or intratracheal (Dong *et al.*, 1996) instillation of transducing compositions to effect transduction of lung cells.

V. Examples

EXAMPLE 1

The infectious hepatitis A virus cDNA clone used for construction of a viral genome encoding a selectable marker was constructed by insertion of cDNA segments cloned from a highly cell culture-adapted variant of hepatitis A virus (HM175/18f, Zhang *et al.*, 1996) into a prototype infectious cDNA obtained from the National Institute of Health (pHAV/7, Cohen *et al.*, 1986). Thus, although almost all of the viral cDNA now in the parent infectious clone is derived from materials previously cloned the construction of this clone was facilitated by use of a NIH infectious clone. However, it should be recognized that the construction of such infectious clones is standard practice in molecular virology and that this could be easily replicated by an investigator with normal skills and knowledge without reference to the NIH infectious clone or NIH technology.

One embodiment of the present invention was forced selection of an HAV HM175 strain variant that is capable of enhanced antigen yield in MRC-5 cells. This was achieved by addition of a sequence encoding a selectable marker (*zeo*) within the HAV polyprotein, at the primary polyprotein cleavage site located between the 2A and 2B coding region (FIG. 1). The specific construction was carried out within a plasmid containing an infectious cDNA copy of the HM175/18f genome (Zhang *et al.*, 1996). The *zeo* coding sequence, amplified PCR™ from

a vector supplied by InVitrogen, was flanked by artificially constructed HAV 3C proteinase cleavage sites, and this cassette was inserted in frame at the normal 2A/2B cleavage site (FIG. 1). A gly-gly hinge was also included in each of the flanking cleavage sites to increase polypeptide chain flexibility and facilitate efficient cleavage. Construction of this cDNA was completed and synthetic RNA derived from 2 sister cDNA constructs was individually transfected into permissive FRh-K4 cells with recovery of viable virus. Virus was recovered from both transfections, as determined by radioimmunofocus assay on BSC-1 cells with foci that were approximately 2-fold smaller than the parent HM175/18f virus foci (in effect small plaque variants, FIG. 1).

As has been found to be the case with poliovirus containing foreign sequences inserted at the 2A/2B junction (Tang *et al.*, 1997), the inventors considered the possibility that the insertion of the *zeo* gene may not be genetically stable. If not, replication of the modified HAV genome could be followed by spontaneous deletion of the *zeo* gene by a process of homologous recombination within the flanking regions (3C cleavage sites). To determine whether this had occurred in virus recovered from these HM175/18f recombinants, viral RNA was subjected to RT/PCR™ using primers that nucleotides larger than that seen from the present virus. This was the only size product observed, confirming successful insertion of the *zeo* gene and its resultant stability within the 2A/2B cleavage site. These PCR™ results were further confirmed by the hybridization of a 32P labeled *zeo* probe specifically to RNA that was isolated from virus recovered from BSC-1 cells infected with HAV containing the *zeo* gene. These results indicate that the HAV genome can tolerate an insertion at the 2A/2B junction without loss of replication competence.

The present invention comprises a more efficient production of viral antigen that would enhance vaccine production, thus lowering unit production costs and potentially increasing vaccine doses produced per cm^2 of cell culture area.

In order to test the possibility that an HAV genome containing the *zeo* gene at the 2A/2B junction could be used to select a variant that has enhanced replication and antigen production properties, HAV containing *zeo* was used to infect BSC-1 cells. Selection of HAV variants was performed by culturing infected cells in the presence of increasing concentrations of the antibiotic Zeocin (50-500 μg/ml). Virus was harvested and its replication potential determined by radioimmunofocus assay in BSC-1 cells. As a control, parallel cultures were infected with parent virus HM175/18f. Cell death was observed at concentrations of

Zeocin >250 μg/ml, however cultures infected with HAV containing the Zeo gene showed greater resistance (*i.e.* increased numbers of viable cells remained for a longer period of time) to Zeocin compared with cultures infected with the parent virus, HM175/18f.

Subsequent radioimmunofocus assays revealed that as the concentration of Zeocin increased, a heterogeneous population of virus variants was selected, with a high proportion of these generating replicating foci larger than that of the virus cultured in the absence of Zeocin. This provides proof of the concept that this system has the potential to isolate HAV variants with an increased replication phenotype within a particular type of cultured cell. The inventors plan to plaque isolate those foci with increased size and determine the genetic basis for increased replication by sequencing cDNA amplified by a combination of reverse transcription and polymerase chain reaction (RT/PCR™). These studies document the utility of this recombinant, zeo-expressing HAV as a tool for selection of new HAV variants with increased replication phenotypes in BS-C-1 cells, and by inference, cell types used for vaccine3 manufacture such as MRC-5 cells.

The primary polyprotein cleavage event in HAV most likely occurs at the 2A/2B junction in contrast to poliovirus in which cleavage occurs at the P1/P2 junction. To investigate whether synthesis of P1-2A could be uncoupled from that of 2BC-P3 we constructed a dicistronic HAV genome containing the EMCV IRES between the 2A and 2B proteins. Insertion of the EMCV IRES did not affect polyprotein processing in vitro. However, transfection of synthetic T7 derived RNA into permissive FRhK-4 cells demonstrated that this RNA had a replication-defective phenotype.

To address this issue, a nucleotide sequence encoding a heterologous protein was introduced into the HAV genome at the 2A/2B site. The gene encoding the selectable marker *zeo (ble)* was flanked by HAV $3C^{pro}$ cleavage sites a placed in-frame between the 2A and 2B proteins (Gln^{836}/Ala^{837}). Replication of HAV RNA containing the *zeo* gene was observed in BSC-1 cells by radioimmunofocus assay with a replication phenotype that was slightly reduced compared to the parent HM175-18f virus. The *zeo* gene was retained for up to 5 passages in BSC-1 cells as determined by RT/PCR. This is the first demonstration that foreign sequence can be inserted at this site and result in a viable virus phenotype. These results confirm that the 2A/2B junction is the primary cleavage site and suggest that other foreign antigens may be engineered at this site in the HAV genome and allow liver specific expression of a foreign gene.

EXAMPLE 2:

The selection HAV viral variants described for BSC-1 cells has been extended to MRC-5 cells, the cells used to manufacture the current HAV vaccine. This preliminary work was conducted essentially the same as that described for the BSC-1 cells. Briefly, MRC-5 cells were infected with HAV containing zeo at the 2A/2B junction and incubated in increasing concentrations of the antibiotic zeocin (50-500ug/ml). As a control parallel cultures were infected with the parent virus HM175/18f. After a period of approximately 2 weeks significant cell death was noted in control cultures at Zeocin concentrations >125ug/ml, however cultures infected with HAV containing zeo showed greater resistance to zeocin with intact cell monolayers at 500ug/ml zeocin. Isolation of viris from these cultures and subsequent radioimmunoifocus assay revealed a heterogenous population of virus variants was selected, however there was not the dramatic increase in focus size noted in the BSC-1 experiment. This may reflect the different viral replication rates between BSC-1 and MRC-5 cells and continuous passage of isolated virus from MRC-5 cells, under zeocin selection may be required to isolate virus variants with enhanced virus phenotypes.

EXAMPLE 3:

The insertion of other foreign coding sequences at the 2A/2B junction has also been explored, in the same context as the *zeo* gene. This has been done to test the size of the insert that can be tolerated by the HAV genome as similar experience with poliovirus has suggested that as you increase the size of the insert the stability of the recombinant virus decreases, resulting in removal of the inserted sequence. The GFP (green fluorescent protein) and the Renilla Luciferase gene have been inserted into the 2A/2B junction of HAV in similar context to that of the zeo gene described above.

Transfection of synthetic T7 derived RNA into permissive FRhK-4 cells demonstrated that this RNA had a viable virus phenotype, albeit handicapped compared to the parent HM175/18f. Stability of the insertion was addressed by RT/PCR using primers that span the insertion. RT/PCR results indicated that insertion of increasingly larger sequences (up to 950bp) within the HAV genome at 2A/2B junction resulted in genetic instability and removal of all or part of the inserted sequence after 3-4 passages. Nevertheless, these results indicate that insertion of foreign sequences of different sizes can result in a viable virus phenotype.

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CLAIMS:

1. A recombinant hepatitis A virus comprising a heterologous nucleic acid sequence.

2. The recombinant hepatitis A virus of claim 1, wherein said nucleic acid sequence is located in a polyprotein region.

3. The recombinant hepatitis A virus of claim 1, wherein said virus is an attenuated virus.

4. The recombinant hepatitis A virus of claim 2, wherein the heterologous nucleic acid sequence is located at the 2A/2B junction.

5. The recombinant hepatitis A virus of claim 4, wherein a gly-gly hinge flanks the heterologous nucleic acid sequence at the 2A/2B junction.

6. The recombinant hepatitis A virus of claim 1, wherein the heterologous nucleic acid sequence comprises a nucleic acid sequence encoding a selectable marker.

7. The recombinant hepatitis A virus of claim 6, wherein the selectable marker is a positive selectable marker.

8. The recombinant hepatitis A virus of claim 7, wherein said positive selectable marker is a drug resistance marker.

9. The recombinant hepatitis A virus of claim 8, wherein the positive selectable marker is a phleomycin resistance gene.

10. The recombinant hepatitis virus of claim 1, wherein the heterologous nucleic acid sequence comprises a nucleic acid sequence for treating a disease or condition.

11. The recombinant hepatitis virus of claim 10, wherein said disease or condition is liver-specific.

12. A method for producing a recombinant hepatitis A virus containing a heterologous nucleic acid sequence comprising:

(a) obtaining a vector containing an infectious cDNA copy of the HAV genome;

(b) cleaving the HAV genome in a polyprotein region; and

(c) inserting a nucleic acid sequence comprising a heterologous nucleic acid sequence into the region.

13. The method of claim 12, wherein said polyprotein region comprises the 2A and 2B coding region.

14. The method of claim 12, further comprising incubating the vector with the insertion under conditions to permit transcription.

15. The method of claim 12, further comprising transfecting the transcribed products into a permissive cell.

16. The method of claim 12, wherein the heterologous nucleic acid sequence is inserted in frame.

17. The method of claim 12, wherein the heterologous nucleic acid sequence further comprises a hepatitis A virus 3C proteinase cleavage site.

18. The method of claim 17, wherein the heterologous nucleic acid sequence further comprises a gly-gly hinge in a flanking cleavage site.

19. The method of claim 17, wherein said heterologous nucleic acid region comprises a selectable marker.

20. The method of claim 12, further comprising the step of transfecting the transcribed product containing the heterologous nucleic acid sequence in the coding region into an HAV permissive cell.

21. A method for screening for a hepatitis A virus variant comprising:

(a) obtaining a recombinant hepatitis A virus comprising a selectable marker conferring resistance to a selectable agent;

(b) infecting an HAV permissive cell with the recombinant HAV; and

(c) exposing the infected cell to the selectable agent.

22. The method of claim 21, wherein the selectable marker is located in a polyprotein region.

23. The method of claim 22, further comprising iterating said exposure at a higher concentration level of said selectable agent.

24. The method of claim 23, further comprising assaying said recombinant HAV for enhanced replication.

25. The method of claim 24, wherein assaying for enhanced replication comprises comparing replication foci of the cell infected with the recombinant HAV and exposed to the selectable agent with either (i) a cell infected with the recombinant HAV but not exposed to the selectable agent or (ii) a cell infected with a vector containing an infectious cDNA copy of the HAV genome and not the selectable marker but exposed to the selectable agent.

26. The method of claim 21, further comprising assaying said recombinant HAV for increased viral antigen production.

27. The method of claim 26, wherein assaying for increased viral antigen production comprises comparing viral antigen production of the cell infected with the recombinant HAV and exposed to the selectable agent with either (i) a cell infected with the recombinant HAV but not exposed to the selectable agent or (ii) a cell infected with a vector containing an infectious cDNA copy of the HAV genome and not the selectable marker but exposed to the selectable agent.

28. The method of claim 26, wherein the viral antigen is derived from HAV.

29. The method of claim 21, further comprising identifying at least one mutation in a candidate variant as compared to the recombinant virus.

30. A hepatitis A virus variant identified by a method of screening comprising:

(a) obtaining a vector encoding a recombinant hepatitis A virus comprising a selectable marker conferring resistance to a selectable agent;

(b) incubating the vector under conditions to permit transcription;

(c) transfecting an HAV permissive cell with the transcribed product; and

(d) exposing the infected cell to the selectable agent.

31. The hepatitis A virus variant of claim 30, wherein the variant is identified by a method of screening further comprising iterating said exposure at a higher concentration level of said selectable agent.

32. The hepatitis A virus variant of claim 31, wherein the variant is identified by a method of screening further comprising assaying said recombinant HAV for enhanced replication.

33. The hepatitis A virus variant of claim 31, wherein the variant is identified by a method of screening further comprising assaying said recombinant HAV for increased viral antigen production.

34. The hepatitis A virus variant of claim 30, wherein said variant contains at least one nucleic acid sequence mutation compared to the recombinant virus.

35. The hepatitis A virus variant of claim 34, wherein said nucleic acid sequence mutation is identified.

36. A method for producing a hepatitis A virus vaccine seed virus comprising:

(a) obtaining a recombinant hepatitis A virus variant comprising a selectable marker;

(b) identifying at least one nucleic acid sequence mutation in the recombinant HAV variant;

(c) obtaining a vector comprising an infectious cDNA copy of the virus;

(d) introducing the nucleic acid sequence mutation of the recombinant HAV variant into the vector;

(e) incubating the mutated vector under conditions to permit transcription;

(e) infecting an HAV permissive cell with the transcribed product; and

(f) incubating the infected cell under conditions to permit viral replication.

37. The method of claim 36, wherein the selectable marker is located in a polyprotein region.

38. The method of claim 36, wherein said permissive cell is an MRC-5 cell.

39. A hepatitis A virus-based vaccine seed virus produced by a method comprising:

(a) obtaining a recombinant hepatitis A virus variant comprising a selectable marker;

(b) identifying at least one nucleic acid sequence mutation in the recombinant HAV variant;

(c) obtaining a vector containing an infectious cDNA copy of the HAV genome;

(d) introducing the nucleic acid sequence mutation of the recombinant HAV variant into the vector;

(e) incubating the mutated vector under conditions to permit transcription;

(f) infecting an HAV permissive cell with the transcribed product; and

(g) incubating the infected cell under conditions to permit viral replication.

40. The hepatitis A virus-based vaccine seed virus of claim 39, wherein said selectable marker is located in a polyprotein region.

41. The hepatitis A virus-based vaccine seed virus of claim 39, wherein the hepatitis A virus variant used in the method to produce the seed virus further comprises a heterologous nucleic acid sequence.

42. The hepatitis A virus vaccine seed virus of claim 41, wherein said variant has enhanced replication.

43. The hepatitis A virus vaccine seed virus of claim 41, wherein said variant has increased viral antigen production.

44. The hepatitis A virus-based vaccine seed virus of claim 41, wherein the heterologous nucleic acid sequence comprises a foreign antigen.

45. The hepatitis A virus-based vaccine seed virus of claim 44, wherein the foreign antigen elicits an immune response.

46. The hepatitis A virus-based vaccine seed virus of claim 45, wherein said foreign antigen is derived from the hepatitis C virus.

47. The hepatitis A virus-based vaccine seed virus of claim 45, wherein said foreign antigen is derived from the hepatitis B virus.

48. A method for producing a recombinant virus comprising:

(a) obtaining a vector comprising a viral genome, wherein said vector comprises a heterologous nucleic acid sequence;

(b) incubating the vector under conditions to permit transcription;

(c) transfecting a permissive cell with the transcribed product; and

(d) incubating the cell under conditions to permit viral replication.

49. The method of claim 48, wherein the heterologous nucleic acid sequence is located in a polyprotein region.

50. The method of claim 49, wherein the viral genome is derived from a positive-strand RNA virus.

51. The method of claim 50, wherein said positive strand RNA virus is a hepatitis C virus.

52. The method of claim 50, wherein the heterologous nucleic acid sequence encodes a selectable marker.

53. A method for producing a viral variant comprising:

(a) obtaining a vector comprising a viral genome, wherein said vector comprises a selectable marker conferring resistance to a selectable agent;

(b) incubating the vector under conditions to permit transcription;

(c) transfecting a permissive cell with the transcribed product; and

(d) exposing the infected cell to the selectable agent.

54. The method of claim 52, wherein said selectable marker is located in a polyprotein region.

55. The method of claim 52, further comprising comprising iterating said exposure at a higher concentration level of said selectable agent.

56. The method of claim 53, further comprising the step of identifying at least one mutation in a candidate variant as compared to a vector not exposed to said selectable agent.

57. The method of claim 56, wherein said nucleic acid sequence mutation is identified.

58. The method of claim 57, further comprising assaying the virus for enhanced replication.

59. The method of claim 56, wherein assaying for enhanced replication comprises comparing replication foci of the cell transfected with the vector and exposed to the selectable agent with either (i) a cell transfected with the vector but not exposed to the selectable agent or (ii) a cell transfected with a vector capable of producing a virus in a permissive cell, wherein said vector comprises nucleic acid sequences of the virus and exposed to the selectable agent.

60. The method of claim 50, further comprising assaying the virus for increased viral antigen production.

61. The method of claim 56, wherein assaying for increased viral antigen production comprises comparing viral antigen production of the cell transfected with the vector and exposed to the selectable agent with either (i) a cell transfected with the vector but not exposed to the selectable agent or (ii) a cell transfected with a vector capable of producing a virus in a permissive cell, wherein said vector comprises nucleic acid sequences of the virus and exposed to the selectable agent.

62. A method for producing a viral vaccine seed virus comprising:

(a) obtaining a viral variant comprising a selectable marker;

(b) identifying at least one nucleic acid sequence mutation in the viral variant;

(c) obtaining a vector vector comprising an infectious cDNA copy of a viral genome used to create the viral variant;

(d) introducing the nucleic acid sequence mutation of the viral variant into the vector;

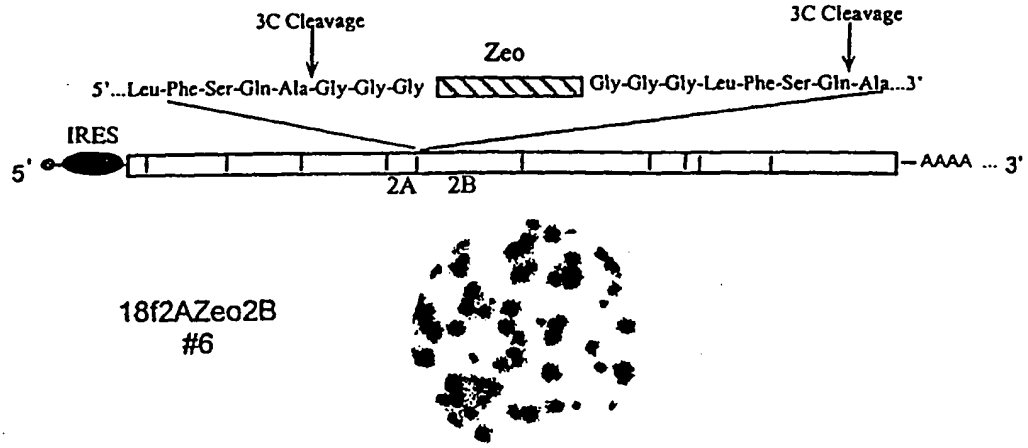
(e) incubating the vector under conditions to permit transcription;

(e) transfecting a permissive cell with the transcribed product; and

(f) incubating the cell under conditions to permit viral replication.

63. The method of claim 62, wherein the viral variant is derived from a positive strand RNA virus.

64. The method of claim 63, wherein the positive strand RNA virus is a hepatitis C virus.


3C Cleavage
3C Cleavage
Zeo
5'...Leu-Phe-Ser-Gln-Ala-Gly-Gly-Gly
Gly-Gly-Gly-Leu-Phe-Ser-Gln-Ala...3'
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AAAA ... 3'
2A
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FIG. 1

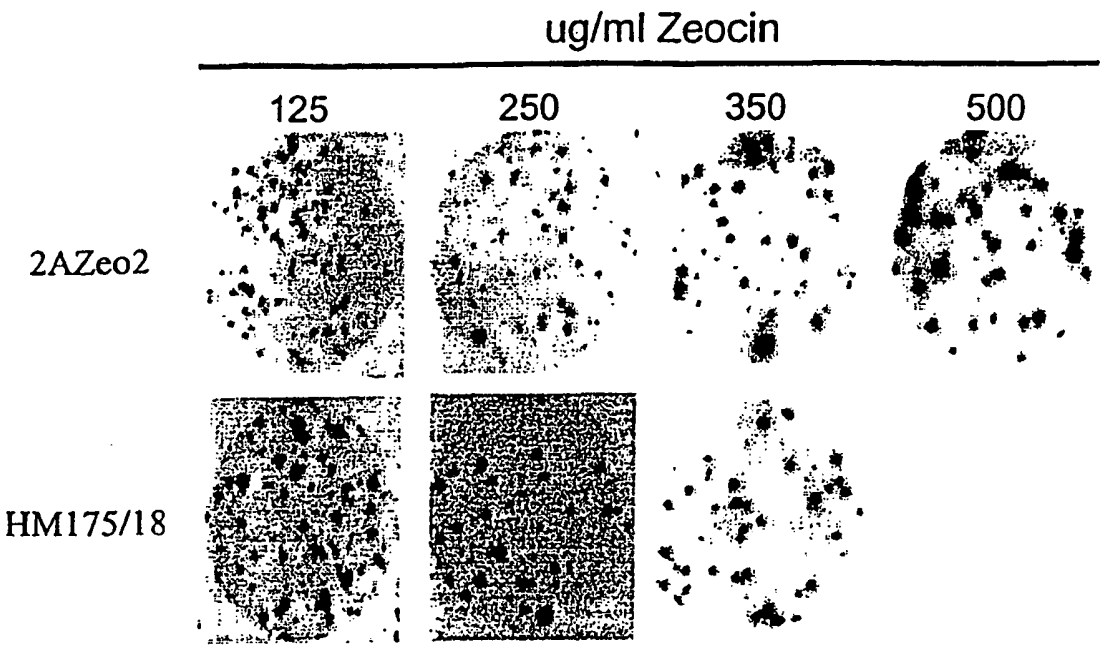

FIG. 2